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Role of a conserved amino-terminal sequence in the ecotropic MLV receptor mCAT1

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Abstract

The amino-terminus of mCAT1 and homologous proteins is predicted to form a positively charged, amphipathic alpha helix on the cytoplasmic side of the plasma membrane. Peptides with similar sequence motifs often provide membrane anchors, protein-protein interaction domains, or intracellular transport-targeting signals. Deleting most of the cytoplasmic N-terminal sequence of mCAT1 led to reduced expression on the cell surface and accumulation in the endoplasmic reticulum but did not abrogate receptor function. Surprisingly, when the N-terminal 36 or 18 amino acids of mCAT1 were fused to green fluorescent protein (gfp), gfp accumulated almost exclusively in mitochondria. Mitochondrial targeting depended on arginines at positions 15 and 16 and was inhibitable by downstream transmembrane sequences. Although the full-length mCAT1 was not detected in mitochondria, the mitochondrial-targeting property of the N-terminal sequence fused to gfp is conserved in orthologous and paralogous proteins that diverged ~80 million years ago, suggesting a conserved biological function. We propose that the conserved N-terminal motif of CAT proteins provides a regulatable signal for transport to, or retention in, different cell membrane compartments.

Keywords: Cationic amino acid transporter; Subcellular localization; Mitochondrial-targeting peptide

Introduction

The receptor for ecotropic murine leukemia virus (MLV)[1] is a polytopic plasma membrane protein designated mCAT1 that functions as a cationic amino acid transporter in a variety of cells (Kim et al., 1991; Wang et al., 1991). Computer analysis of protein structure based on hydrophobicity and lack of an amino-terminal signal peptide sequence suggests that mCAT1 has 14 transmembrane segments with amino- and carboxyl-termini in the cytoplasm (Fig. 1A) (Albritton et al., 1989). mCAT1 is a member of a protein family with at least two paralogous genes in rodents, mCAT2 and mCAT3, approximately 60% identical at the amino acid level. Orthologous human genes with about 90% amino acid identity to mouse CAT1 and CAT2 have been identified (Albritton et al., 1992; Closs et al., 1997; Yoshimoto et al., 1991), along with several more distantly related members of the cationic amino acid transporter family.

We previously reported that mCAT1 is concentrated in cholesterol-rich plasma membrane domains termed rafts, where it interacts with caveolin, a protein involved in non-clathrin-mediated endocytosis. Cholesterol depletion, which disrupts rafts, inhibited MLV entry (Lu and Silver, 2000). The amino terminus of mCAT1 begins with the sequence methionine-glycine-cysteine. In several other proteins this sequence serves as a raft-targeting signal due to myristoylation of the glycine and palmitoylation of the cysteine (Shenoy-Scaria et al., 1994).

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[1] *Abbreviations used:* CAT, cationic amino acid transporter; env, envelope; GFP, green fluorescent protein; hCAT, human CAT; mCAT, mouse CAT; MLV, murine leukemia virus; MTP, mitochondrial-targeting peptide; PM, plasma membrane.

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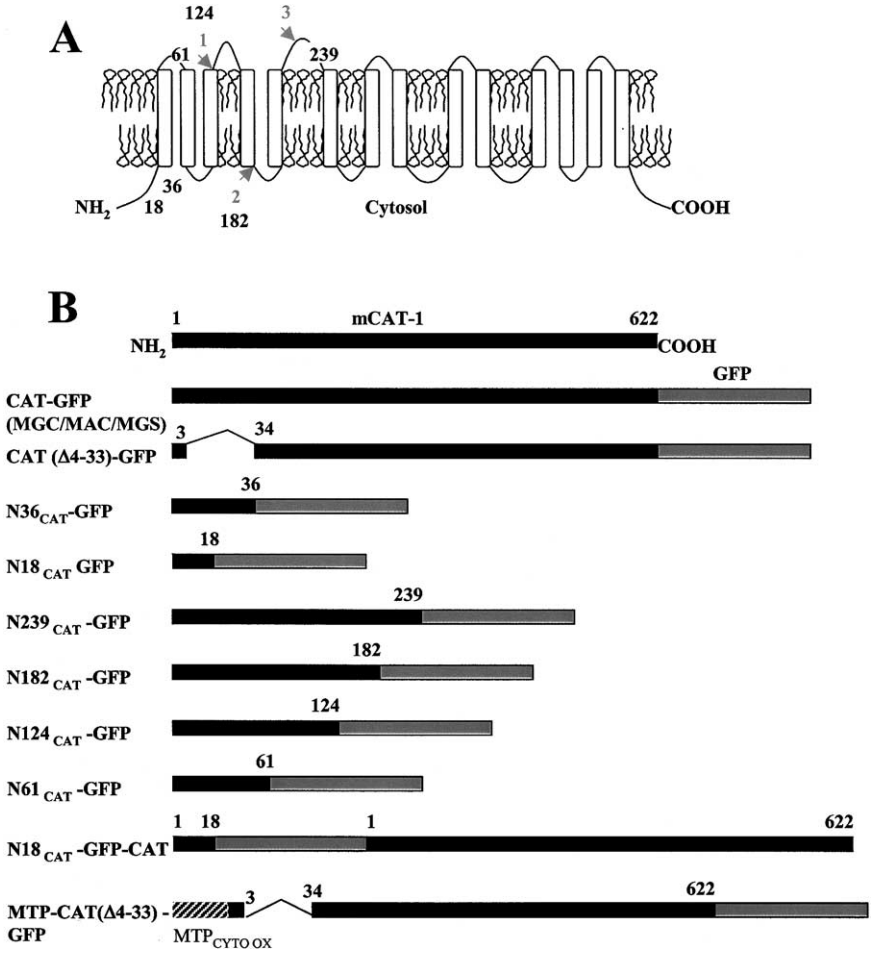

Fig. 1. Structure of mCAT1 protein molecule and chimeric constructs. (A) Predicted membrane topology of mCAT1. Black numbers refer to amino acid position starting from the amino terminus. Gray arrows indicate putative boundaries of the first, second, and third exons. (B) Structure of chimeric mCAT1 and GFP genes. Wild-type or mutant mouse or human CAT1 coding sequences were fused in frame to the N- or C-terminus of GFP. Black box, CAT1; gray box, GFP; cross-hatched, mitochondria-targeting sequence of subunit VIII of cytochrome *c* oxidase. See text for details.

Downstream of the met-gly-cys motif, but within the presumed first intracytoplasmic portion of mCAT1, are several basic residues and two additional cysteines. In other N-terminally acylated proteins, adjacent basic domains or palmitoylated cysteines contribute to targeting to particular membranes (Chen et al., 2001; Ono et al., 2000). The N terminal 36 amino acids of mCAT1 are predicted to form an amphipathic alpha helix, a motif involved in protein-protein and protein-membrane interactions as a consequence of hydrophobic and hydrophilic surfaces on opposite sides of the alpha helix. Given the potential for interaction with other proteins and membranes, it was of interest to investigate whether the N-terminal sequence of mCAT1 played a role in its intracellular localization. We report here that the N-terminal 36 amino acid segment of mCAT1 affects its association with specific cellular membranes including, unexpectedly, mitochondria, in a manner that is sensitive to adjacent transmembrane sequences.

Results

To evaluate the intracellular localization of mCAT1, we used a fusion construct in which GFP is fused in frame to the cytoplasmic C-terminus of mCAT1. We and others previously showed that this construct is fully functional as a murine leukemia virus receptor (Lee, et al., 1999; Lu and Silver, 2000; Masuda et al., 1999). When expressed via a CMV-promoter in the DNA expression vector (pEGFP-N1) in HEK293 cells, mCAT1-GFP localized almost exclusively on the plasma membrane (Fig. 2A). In BHK cells, mCAT1-GFP was largely on the plasma membrane with some fluorescence in the perinuclear area, using either the DNA expression vector or a Sindbis expression vector (Fig. 2B). This pattern of fluorescence is consistent with a protein that transits from the ER to Golgi to plasma membrane. Previously published Western blot analysis showed that most of the protein that reacted with anti-GFP antibody was of the size expected for the chimeric mCAT1-GFP fusion pro-

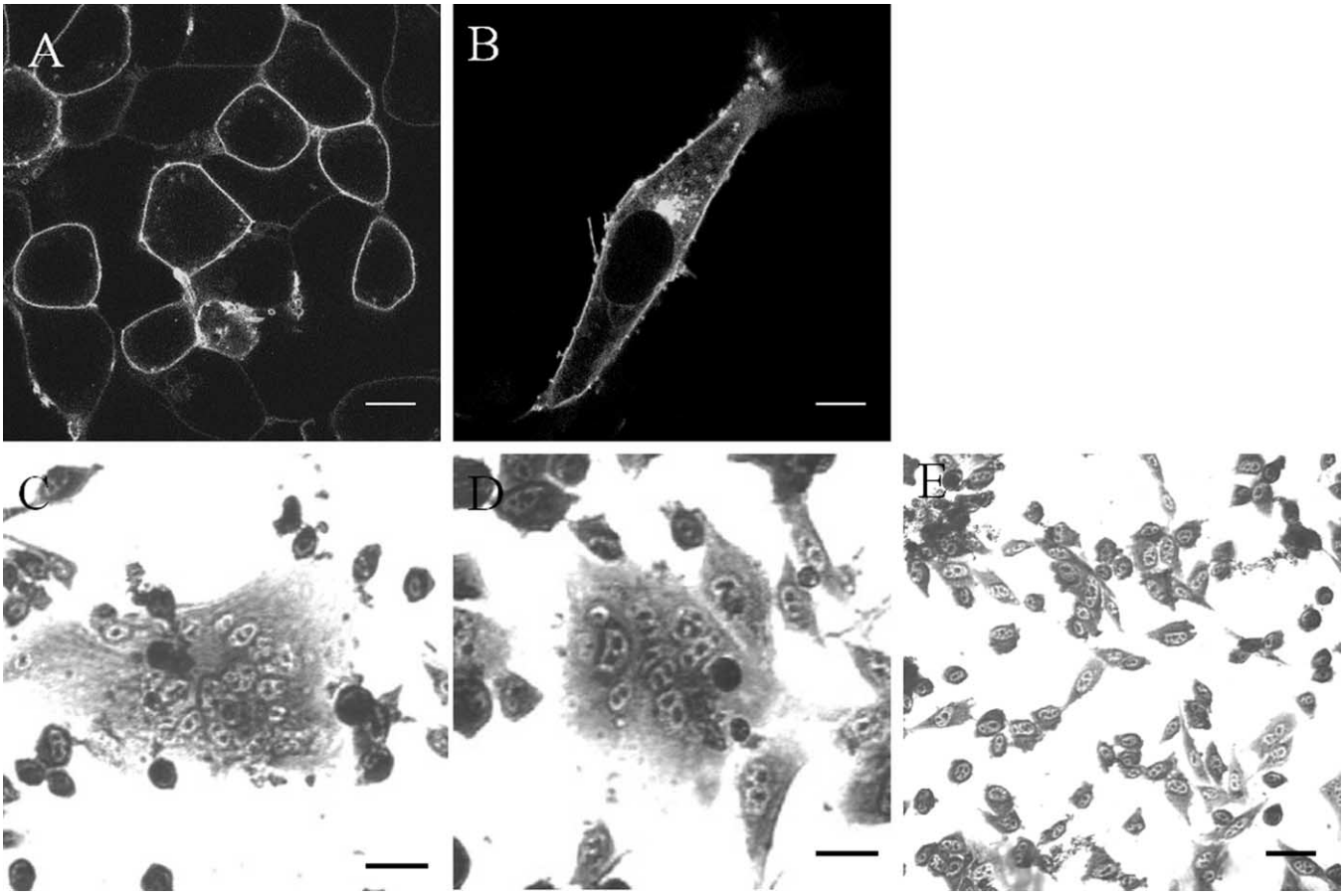

Fig. 2. Subcellular localization and syncytia forming activity of wild-type and mutant mCAT1-GFP. Transfected cells were visualized by fluorescence confocal microscopy or stained with carbol fuchsin/methylene blue. (A) HEK293 cells transfected with wild-type mCAT1-GFP. (B) BHK cells transfected with wild-type mCAT1-GFP. (C) BHK cells transfected with wild-type mCAT1-GFP, and cultured with BHKenv cells. (D) BHK cells transfected with mCAT1(MAC)-GFP and cultured with BHKenv cells. mCAT1(MGS)-GFP (not shown) is same as mCAT1(MAC)-GFP. (E) Nontransfected BHK cells cultured with BHKenv cells. Bars in A and B, 5 μm; in C and D, 20 μm.

tein (Lee et al., 1999; Lu and Silver, 2000), so the fluorescence should be a representative of the intracellular localization of the mCAT1-GFP fusion protein.

The met-gly-cys motif at the N-terminus of mCAT1 has little or no effect on its subcellular localization

To see if the met-gly-cys motif at the amino terminus of mCAT1 affected subcellular localization, we mutated this sequence to met-ala-cys or met-gly-ser. The mutation of cysteine to serine blocks palmitoylation, and substitution of alanine for glycine blocks both fatty acylation steps (Wang et al., 1999). There was no difference between wild type and mutants in terms of subcellular localization (not shown). Both wild-type and mutant mCAT1-GFP were associated with rafts, as evidenced by their resistance to cold Triton X-100 treatment (not shown). The mutant versions of mCAT1 were indistinguishable from wild type in ability to form syncytia when cocultivated with cells expressing a fusogenic from of murine leukemia virus envelope (Figs. 2C and D). Thus, the met-gly-cys sequence does not affect subcellular localization or fusogenicity of mCAT1-GFP with murine leukemia virus envelope.

The cytosolic amino terminus of mCAT1 facilitates transport to the plasma membrane

To investigate the role of other portions of the cytosolic amino terminus of mCAT1 on membrane localization, we deleted amino acids 4 through 33 in the wild-type and met-gly-cys-mutant mCAT1-GFP expression vectors. In BHK cells transfected with mCAT1-GFP fusion constructs in which amino acids 4–33 were deleted (mCAT1(Δ4–33)), much of the GFP fluorescence was found in reticular structures rather than plasma membrane (Fig. 3A). Cotransfection with a DsRed fusion gene containing an ER retention signal (pDsRed2-ER; Clontech) showed that these structures were ER (Figs. 3B and C). Similar results were obtained with Δ4–33 constructs starting with met-ala-cys and met-gly-ser (not shown). The arrest in the ER was not complete, however, because a low level of mCAT1-GFP was detected on the PM, not colocalized with DsRed2-ER

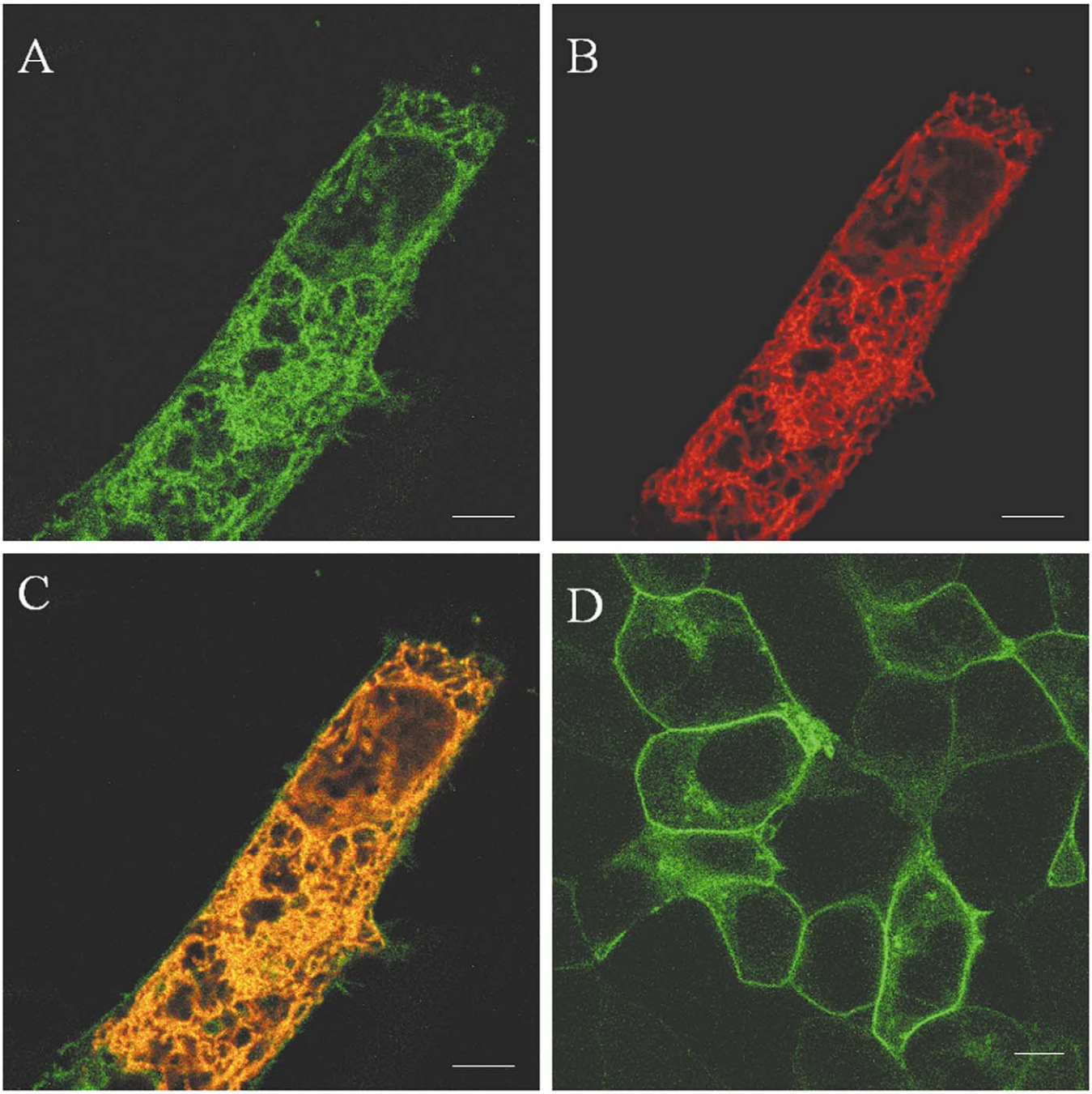

Fig. 3. Deletion of the N-terminus of mCAT1 leads to partial arrest in the ER. mCAT 1(Δ4–33)-GFP was transfected into BHK and HEK293 cells, with or without pDsRed2-ER cotransfected, respectively. (A) mCAT1(Δ4–33)-GFP in a BHK cell. (B) pDsRed2-ER in the same BHK cell. (C) Merge of A and B. (D) mCAT1(Δ4–33)-GFP in HEK293 cells. Bars, 5 μm.

(Fig. 3C), and syncytia were detected when BHK cells transfected with the deletion constructs were cocultivated with murine leukemia virus envelope-expressing cells (not shown). Furthermore, most of the GFP fluorescence was localized on the PM when HEK293 cells were transfected with the deletion constructs (Fig. 3D). Thus, deletion of most of the N-terminal cytosolic domain leads to partial arrest in the ER in a cell-type-dependent fashion.

The N-terminus of mCAT1 encodes a mitochondrial-targeting peptide signal (MTP)

To evaluate the membrane-targeting function of the N-terminus of mCAT1 further, we fused the first 36 amino acids of mCAT1 to GFP in the vector pEGFP-N1. This caused GFP, which is normally distributed uniformly in the cytoplasm (Fig. 4A), to accumulate in filamentous struc-

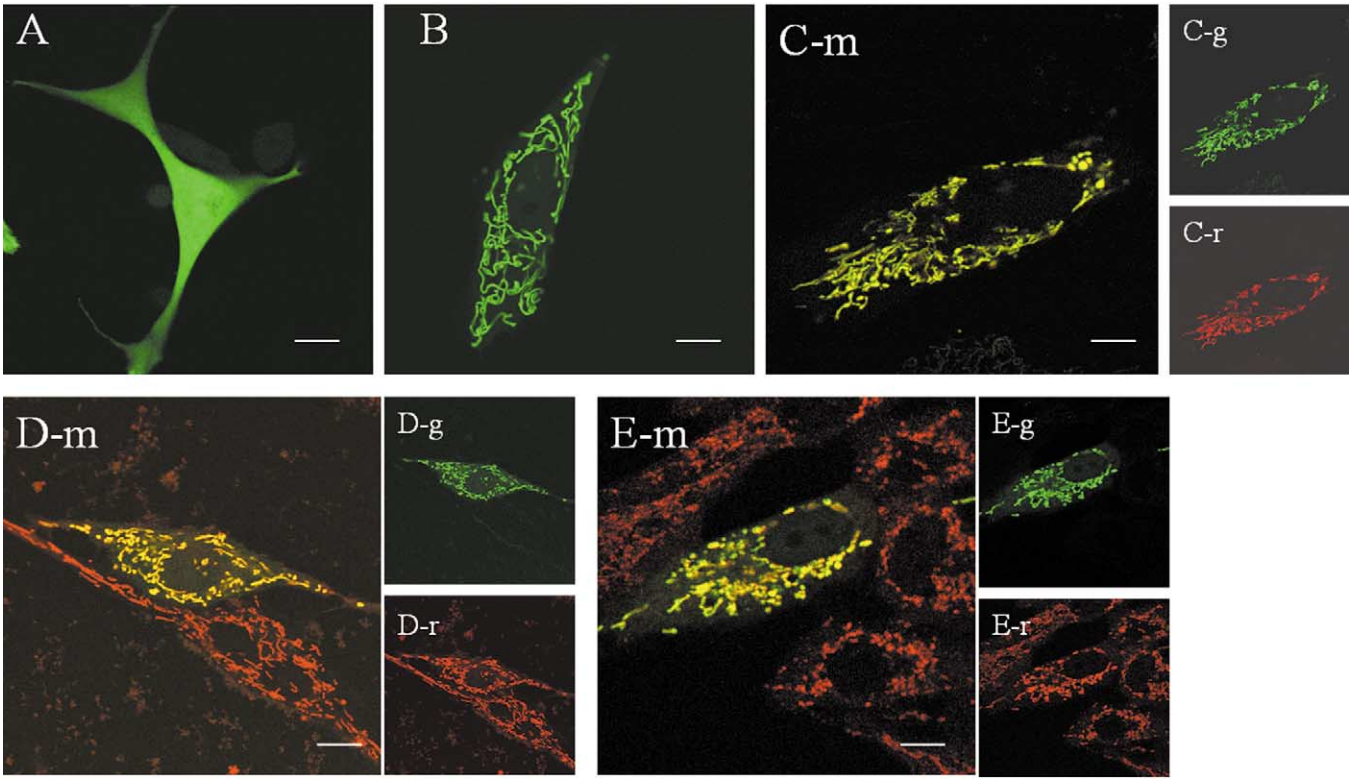

Fig. 4. Subcellular localization of $N36_{mCAT1}$-GFP and colocalization with mitochondrial markers. BHK cells were transfected with GFP, $N36_{mCAT1}$-GFP and/or MTP-DsRed2 and visualized by fluorescence confocal microscopy. For colocalization images, g refers to green emission, r to red emission, and m to the merged image in which dual emission is displayed in yellow. (A) Plain EGFP. (B) $N36_{mCAT1}$-GFP. (C) $N36_{mCAT1}$-GFP and MTP-DsRed2 cotransfected. (D) Cells transfected with $N36_{mCAT1}$-GFP and stained with MitoTracker Red. (E) Cells transfected with $N36_{mCAT1}$-GFP and stained with antibody to cytochrome oxidase subunit I, followed by Cy5-conjugated secondary antibody. In D and E all cells are stained but only an occasional cell is transfected. Bars, 5 μm.

tures (Fig. 4B). Since mitochondria can also appear filamentous (Griparic et al., 2001), we compared the fluorescence pattern of cells transfected with $N36_{mCAT1}$-GFP with that of cells transfected with pEYFP-Mito, a plasmid that encodes a mitochondrial-targeting sequence from cytochrome *c* oxidase fused to the N-terminus of YFP; the patterns were indistinguishable. To confirm this, we transferred the MTP from cytochrome *c* oxidase to pDsRed2-N1, a plasmid encoding a red fluorescent protein that can be distinguished spectroscopically from EGFP (Mizuno et al., 2001). BHK cells transfected with MTP-DsRed2 showed the same filamentous structures in red (Fig. 4C-r), and the red and green fluorescence colocalized perfectly in cells cotransfected with MTP-DsRed2 and $N36_{mCAT1}$-GFP (Fig. 4C-m). Mitochondrial targeting was confirmed by staining BHK cells transfected with $N36_{mCAT1}$-GFP with MitoTracker Red (Molecular Probes), a vital dye that specifically stains mitochondria (Fig. 4D-r). Again, the red and green fluorescence colocalized perfectly (Fig. 4D-m). We also stained BHK cells transfected with $N36_{mCAT1}$-GFP with a monoclonal antibody to the mitochondrial enzyme cytochrome oxidase subunit I followed by Cy5-labeled secondary antibody (Fig. 4E-r). The Cy5 and GFP fluorochromes colocalized (Fig. 4E-m), confirming that the 36 N-terminal amino acids from mCAT1 redirected GFP to mitochondria.

Computer prediction of a mitochondrial-targeting motif in the amino terminus of mCAT1

Mitochondrial-targeting peptides do not share a consensus sequence but are believed to form amphipathic alpha helices with basic amino acids on one face, and generally lack acidic amino acids. Due to the lack of a common sequence or sequence motifs, computer programs based on neural network principles have been developed for predicting MTPs. We used one such program, TargetP v1.01 at http://www.cBS.DTU.DK/services/TargetP/ (Emanuelsson et al., 2000) to analyze the amino terminus of mCAT1 and related proteins. TargetP predicted that the N-terminal 36 amino acids of mouse CAT1 constituted a mitochondrial-targeting peptide. Most of the information for mitochondrial targeting was apparently contained in the first 18 amino acids of mCAT1 since TargetP predicted that the N-terminal 18 amino acids of mCAT1 fused to GFP would also localize in mitochondria. We confirmed this prediction by confocal examination of BHK cells transfected with the correspond-

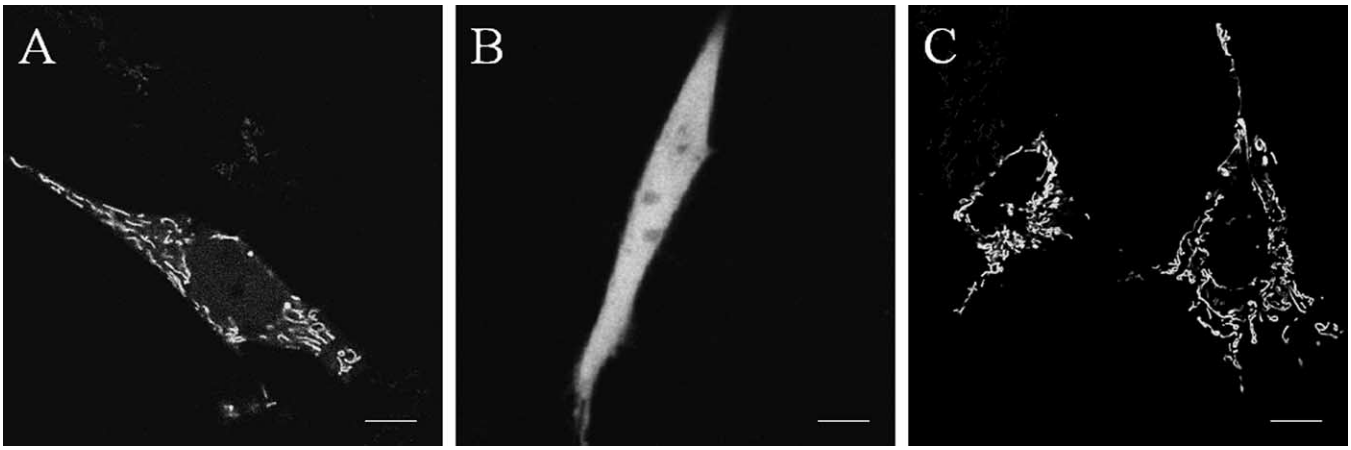

Fig. 5. Subcellular localization of RR-EE mutant and human $N18_{CAT1}$-GFP. BHK cells were transfected with wild-type or mutant mouse $N18_{mCAT1}$-GFP or human $N18_{hCAT1}$-GFP and observed by fluorescence confocal microscopy. (A) Wild-type mouse $N18_{mCAT1}$-GFP. (B) Mutant mouse $N18(RR\text{-}EE)_{mCAT1}$-GFP. (C) Human $N18_{hCAT1}$-GFP. Bars, 5 μm.

ing construct, $N18_{mCAT1}$-GFP (Fig. 5A). The green fluorescence of $N18_{mCAT1}$-GFP colocalized with the red fluorescence of MitoTracker Red and MTP-DsRed2 in BHK and XC cells (not shown). Further analysis of the mitochondrial-targeting signal by TargetP indicated that any of the N-terminal 18 amino acids of mCAT1 could be replaced by glycine without altering predicted mitochondrial localization of $N18_{mCAT1}$-GFP, with the exception of the two arginines at positions 15–16. To evaluate the role of these arginines, we changed them to negatively charged glutamic acids (EE). Transfection of BHK cells with $N18(RR\text{-}EE)_{mCAT1}$-GFP led to uniform cytosolic fluorescence like EGFP (Fig. 5B), indicating that these arginines, or possibly basic rather than acidic residues at these positions, are crucial for mitochondrial targeting.

The mCAT1(1–18) mitochondrial-targeting signal has been conserved over evolutionary time scales

The first 18 amino acids of the human CAT1 homolog differ in three positions from the sequence of mouse CAT1 (Table 1). TargetP predicted that the human sequence fused to GFP would also target mitochondria. To see if this were the case, we constructed $N18_{hCAT1}$-GFP. This plasmid induced the same mitochondrial pattern of fluorescence (Fig. 5C) as the mouse version (Fig. 5A). We also tested a construct with the mouse CAT3 sequence fused to EGFP, which also localized to mitochondria. Table 1 compares the amino-terminal sequences of murine and human CAT genes predicted by TargetP to contain N-terminal mitochondrial-targeting peptides. Phylogenetic analysis shows that the CAT1, CAT2, and CAT3 genes are more distantly related to each other than each mouse gene is to its human homolog. Three other CAT-related genes in GenBank were predicted by TargetP to contain amino-terminal MTPs: Reference Nos. gi14763945, gi11417815, and gi9790235.

The first exon of mCAT1 can override the mitochondrial-targeting peptide signal

Since the mitochondria-staining pattern was not seen in cells transfected with the full-length mCAT1-GFP (Figs. 2A and B), some feature of full-length mCAT1 must override the amino-terminal MTP. To identify this feature, we made progressive C-terminal deletions in mCAT1 sequences placed upstream of GFP in pEGFP-N1. Constructs encoding 239, 182, or 124 N-terminal amino acids from CAT1 led to predominantly reticular

Table 1
Comparison of amino-terminal sequences of CAT genes

Name	Sequence	Sequence Ref. gi No.
mCAT1	*M***G***C*K**N***LL***G**L*G***Q**Q*M*L*RRKVV*D**CS**-R***EE*S*RL*S*RC*L*N*T***Y***D*	1706186
hCAT1	*M***G***C*K*VLL***N**I*G***Q**Q*M*L*RRKVV*D**CS**-R***EE***TRL*S*RC*L*N*T*F*D*	1706185
mCAT3	*M***LW***Q**A**L**RR**FG**Q**K**L*V*RR*R*V*LE**L**G**-M**G***ETRLA*RCLSTL*D*	17989406
hCAT3	*MP***W***Q**A*F**RR**FG**Q**K**L*V*RR*RTLE**SG**-M**A***ETRLA*RCLSTL*D*	1575776
mCAT2	**M***I**PC**R**A**V**L**T**FA**R**C**L**I**RRK**I**V**T**L**DS**L**E**DSK**L**C**RCLSTM**D*	1706189
hCAT2	**M***I**PC**R**A**AL**T**FA**R**C**L**I**RRK**I**V**T**L**DS**L**E**D**T**K**L**C**RCL**T**T**V**D*	1706188
Consensus	MPCKALL FGQ LIRRKVVDL LEETRLARCLSTLD	

Amino acid code: italic, same amino acid as consensus; bold, nonsimilar amino acid; underlined, similar amino acid.

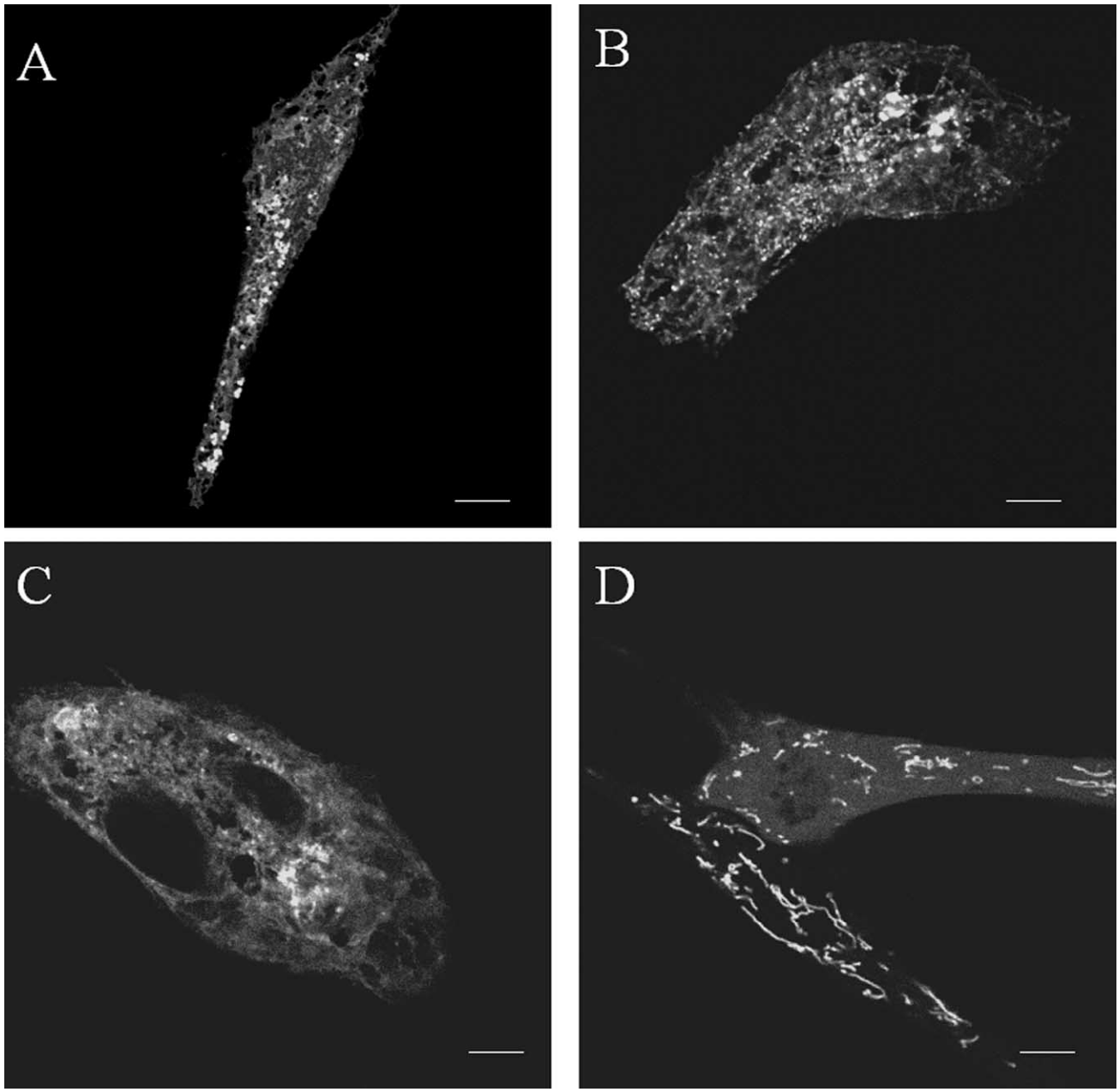

Fig. 6. N-terminal sequences longer than 61 amino acids of mCAT1 override the MTP. BHK cells were transfected with different GFP chimeras and visualized by fluorescence confocal microscopy. (A) $N239_{mCAT1}$-GFP. (B) $N182_{mCAT1}$-GFP. (C) $N124_{mCAT1}$-GFP. (D) $N61_{mCAT1}$-GFP. Bars, 5 μm.

GFP fluorescence (Figs. 6A–C), whereas the amino-terminal 61 amino acids led to mitochondrial fluorescence (Fig. 6D), which colocalized with MitoTracker Red (not shown). Amino acids 1–182 correspond to the first two exons of mCAT1, whereas amino acids 1–124 should contain only the first exon, assuming conservation of exon boundaries between mouse and man (Yoshimoto et al., 1991). Thus, the first exon alone, which encodes the first three putative transmembrane segments (see Fig. 1A), overrides the MTP, while amino acids 1–61, which include only the first transmembrane segment, do not.

Subcellular localization of mCAT1 chimeras containing GFP inserted near the amino terminus or in which the N-terminal sequence is replaced with a bone fide mitochondrial-targeting peptide

To rule out the possibility that full-length mCAT1-GFP might not have been detected in mitochondria due to proteolytic cleavage and loss of the carboxy-terminal GFP marker, we placed GFP upstream of mCAT1 but downstream of the N18 MTP in the construct $N18_{mCAT1}$-GFP-mCAT1 (Fig. 1B). This vector also resulted in reticular

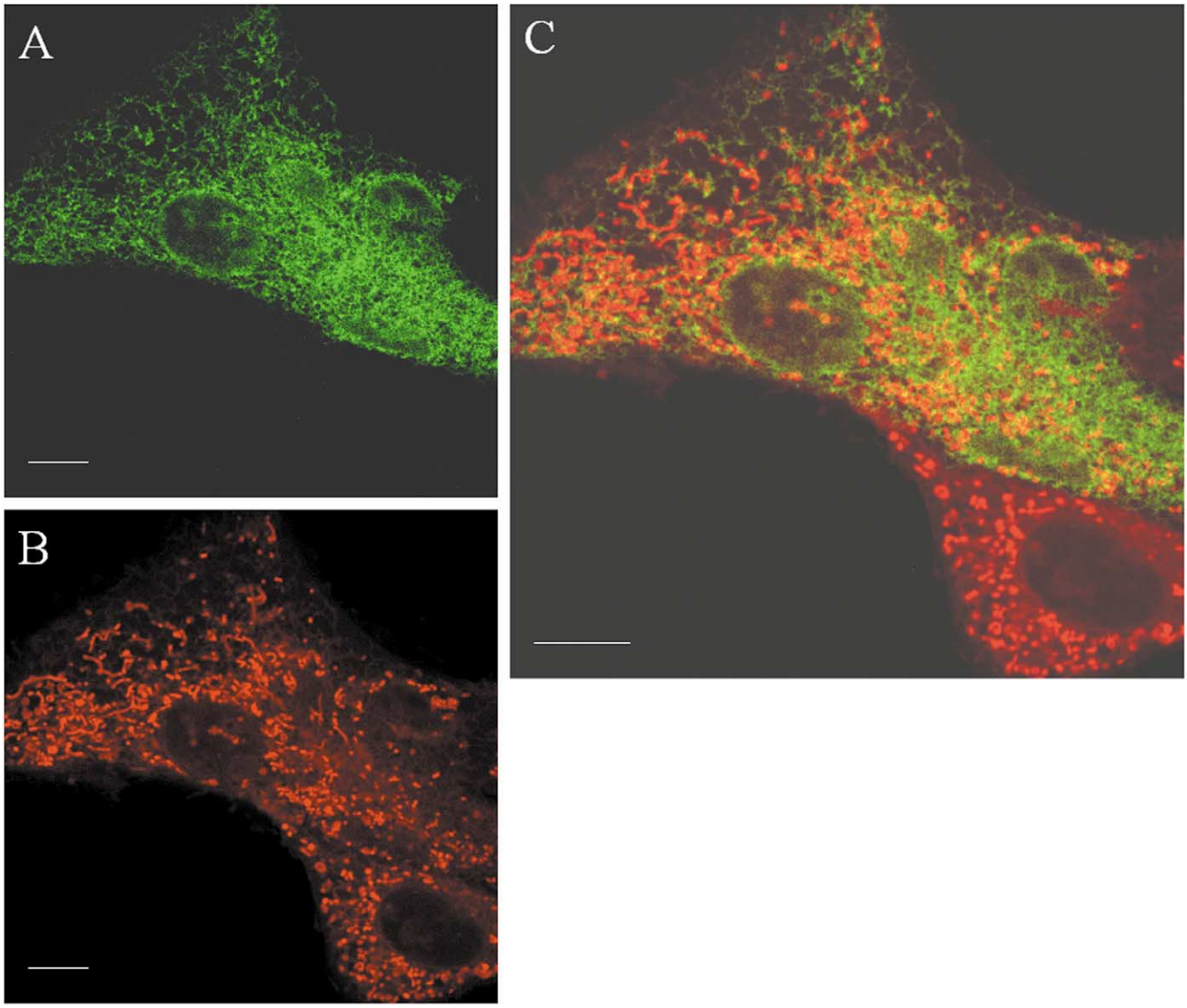

Fig. 7. Non-colocalization of $N18_{mCAT1}$-GFP-mCAT1 and MitoTracker Red. BHK ells were transfected with $N18_{mCAT1}$-GFP-mCAT1, cocultured with BHKenv cells, stained with MitoTracker Red, and visualized by fluorescence confocal microscopy. (A) GFP fluorescence in syncytium. (B) MitoTracker Red emission of same field. (C) Merge of A and B. Bars, 3 μm.

fluorescence that did not colocalize with mitochondria (Fig. 7), and led to syncytia on cocultivation of transfected cells with murine leukemia virus envelope-expressing cells. To see if the N-terminal MTP of mCAT1 was peculiar in its ability to be overridden by downstream sequences, we put a bona fide mitochondrial-targeting sequence from subunit VIII of cytochrome *c* oxidase (Rizzuto et al., 1989) at the amino-terminus of mCAT1(Δ4–33)-GFP, in a construct designated MTP-mCAT1(Δ4–33)-GFP. In most cells, fluorescence was detected on the PM or in a reticular pattern that did not colocalize with mitochondria (Figs. 8A and B). In rare cells (less than 0.1% of transfected cells examined), the cytochrome oxidase MTP redirected mCAT1(Δ4–33)-GFP to mitochondria as judged by a mitochondrial fluorescence pattern that colocalized with MitoTracker Red (Fig. 8C). We conclude that the natural MTP sequence at the amino-terminus of mCAT1 is not peculiar in being overridden by downstream mCAT1 sequences, and these sequences have a potent, though not absolute, ability to override the targeting ability of a bona fide MTP.

Discussion

Conservation of amino-terminal, mitochondrial-targeting sequences in the mouse and human CAT genes over the roughly eighty million years since mouse and man diverged suggests that these sequences have functional significance. The gene duplication events giving rise to the CAT paralogues are older than the man-mouse divergence since their

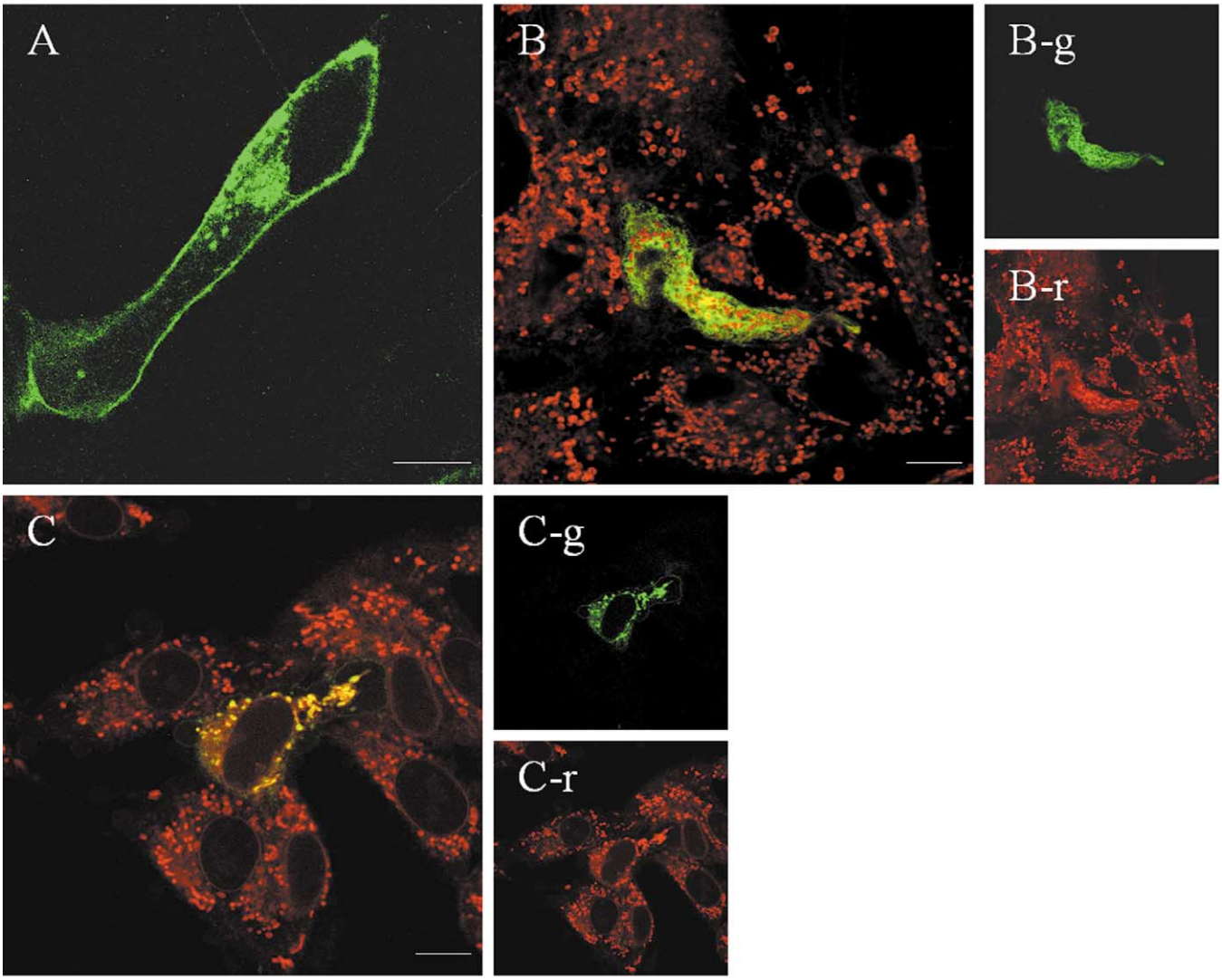

Fig. 8. Subcellular localization of MTP-mCAT1(Δ4–33)-GFP and rare colocalization with mitochondrial markers. BHK cells were transfected with MTP-mCAT1(Δ4–33)-GFP, stained by MitoTracker Red, and visualized by fluorescence confocal microscopy. For colocalization images, g refers to green emission, r to red emission, and m to the merged image in which dual emission is displayed in yellow. (A) typical cell transfected with MTP-mCAT1(Δ4–33)-GFP showing membrane fluorescence. (B) Cell transfected with MTP-mCAT1(Δ4–33)-GFP showing reticular fluorescence that does not colocalize with mitochondria. (C) Rare cell showing colocalization of MTP-mCAT1(Δ4–33)-GFP with MitoTracker Red. Bars, 5 μm.

sequences are more distantly related to each other than to their human homologues and they are present in both species; thus, they provide even stronger evidence for selective pressure to retain the MTPs. The fact that human CAT2, the amino-terminal sequence of which is most divergent, is not predicted to contain an MTP shows that the amino-terminal sequences of the other CAT genes could have diverged. MTPs would not have been retained to preserve murine leukemia virus receptor function, since leukemia viruses are deleterious in mice and deleting the MTP in mCAT1 did not prevent MLVenv-mediated syncytia (Fig. 2C), nor are MTPs predicted to be present in the related, type C retrovirus receptors Pit-1 and Pit-2. Presumably, retention of a mitochondrial-targeting sequence is important for some normal cellular function of CAT.

One possible function is targeting a portion of CAT molecules to mitochondria. Mitochondria import cationic amino acids for intramitochondrial protein synthesis (Keller, 1968). Partly for this reason, mCAT1 was initially considered a candidate gene for an inherited metabolic disease of mitochondria (Albritton et al., 1992). Also, mCAT1 forms a complex with nitric oxide synthase on the plasma membrane of some cells (McDonald et al., 1997), and nitric oxide synthase is found in mitochondria (Tatoyan and Giulivi, 1998; López-Figueroa et al., 2000), so mCAT1 might be important for mitochondrial nitric oxide synthesis. If the N-terminus is a functional MTP, the fact that we did not detect mCAT1-GFP in mitochondria by confocal microscopy could be due to the amount of mCAT1 in mitochondria being too low to detect above a background of GFP in other organelles, or because the mitochondrial-targeting sequence might be active under different physiological conditions or

in cell types that were not tested in vitro. Some MTPs act in a conditional manner following removal of overriding signals by proteolytic cleavage, alternative splicing, use of downstream translation sites (Addya et al., 1997; Anandatheerthavarada et al., 1999; Emr et al., 1986; Little et al., 2001; Neve and Ingelman-Sundberg, 2001), or conformational changes induced by altered pH, phosphorylation, or interaction with other proteins (Gudi et al., 1997; Hebert and Matera, 2000; Nakai and Ishikawa, 2000; Pedraza et al., 1997; Watanabe et al., 2001).

The fact that a few cells transfected with MTP-mCAT1(Δ4–33)-GFP contained GFP in mitochondria (Fig. 8C) is consistent with the hypothesis that under some conditions, an N-terminal MTP directs mCAT1 to mitochondria. However, we were not able to identify conditions under which mitochondrial localization was more than an extremely rare event. We tested the wild-type mCAT1-GFP and/or MTP-mCAT1(Δ4–33)-GFP constructs in human 293 cells, BHK cells, XC cells, and a mouse pulmonary cell line, MTCC2, that might be considered a better candidate for mitochondrial localization of mCAT1 because its mitochondria contain caveolin (Li et al., 2001). We tried activating mitochondria in transfected cells by treatment with thyroid hormone T3 (Wrutniak-Cabello et al., 2001), heating to 41°C, cotransfection with an expression vector for heat shock protein 70, and inducing apoptosis with staurosporine, a treatment that causes the Bax protein to translocate from the cytoplasm to mitochondria (Nechushtan et al., 2001). None of these treatments led to visually detectable relocation of mCAT1-GFP and/or MTP-mCAT1(Δ4–33)-GFP to mitochondria. Therefore, we do not rule out the possibility that the N-terminal sequence of mCAT1 does not function as a mitochondria targeting peptide in the context of full-length mCAT1.

Another possibility is that the N-terminus of mCAT1 is a remnant of what was once a functional MTP in the evolutionary past. A cryptic MTP at the N-terminus of peroxisomal alanine-glyoxylate aminotransferase provides a precedent for this phenomenon (Purdue et al., 1991). If the MTP is an evolutionary remnant, it may have no relevance for mCAT1's current role in amino acid transport.

A third possibility, and one we favor, is that the N-terminal sequence of mCAT1 has a different function in the context of the full-length protein that is related to its ability to target gfp to mitochondria. Such a function would likely involve affinity of the N-terminal amphipathic helix for membrane, hydrophobic protein, or negatively charged species. The mitochondrial transport pathway involves sequential binding of the MTP to proteins rich in acidic amino acids in the cytoplasm and then in the outer and inner mitochondrial membranes ("acid chain hypothesis," Truscott et al., 2001). Transfer across mitochondrial membranes is thought to require that the transported protein remain in an unfolded state as there is an inverse correlation between folding kinetics and transport (Zhou and Wiener, 2001), and chaperones that unfold or retain proteins in an unfolded state can promote mitochondrial import (Voos et al., 1999). The first three transmembrane domains of mCAT1 may block transport into mitochondria by folding rapidly into a nontransportable conformation or by causing mCAT1 to insert in the ER, whereas the first transmembrane segment is insufficient for ER membrane insertion. But the orientation of mCAT1 in the ER, Golgi, or PM would leave the MTP in a position to interact with cytoplasmic proteins, cytoplasmic portions of membrane-associated proteins, or negatively charged lipids such as phosphoserine and phosphotidylinositol on the inner surface of the PM. We propose that this interaction promotes the transport of mCAT1 to the PM, or its retention there, as observed in BHK cells. Further work is necessary to identify potential binding partners of the N-terminal sequence when attached to gfp and in the context of full-length mCAT1.

Materials and methods

DNA constructs

GFP chimeras of wild-type and mutant mCAT1 used in this study are diagrammed in Fig. 1B. Polymerase chain reaction (PCR) was used to mutate the MGC motif at the very N-terminus of mCAT1 to MAC or MGS, and to delete the 30 amino acids between the MGC motif and the first transmembrane domain of mCAT1, designated mCAT1(MAC/MGS)-GFP and mCAT1(Δ4–33)-GFP, respectively. The downstream primers for these three constructs were the same: 5′-ACC AAA CTC GCC GTA GCA CAG GC-3′. The upstream primers with *Xba*I site (italics) and mutations (bold) were 5′-GTT G*TC TAG A*CA CCA CCA TGG **CA**T GCA AAA ACC TGC TCG GT-3′, 5′-GTT G*TC TAG A*CA CCA CCA TGG G**A**T **C**CA AAA ACC TGC TCG GT-3′, and 5′-GTT G*TC TAG A*CA CCA CCA TGG G**A**T GCC TCA ACA CCT ATG ACC-3′, respectively. PCR products were cut with *Xba*I/*Fse*I and cloned into a Sindbis expression vector containing mCAT1-GFP (Kazachkov et al., 2000) cut with the same enzymes. Mutations were identified by restriction digest using introduced sites specific for the mutated sequences (underlined). To express wild-type or mutant mCAT1-GFP chimeras via a DNA expression vector, the mCAT1-GFP gene was cut from the Sindbis vector with *Bam*HI/*Not*I and substituted for GFP in pEGFP-N1 (Clontech, Palo Alto, CA) cut with *Bgl*II/*Not*I. Oligonucleotide adapters encoding the N-terminal 36 or 18 amino acids of mCAT1 were inserted in frame at the *Age*I site of pEGFP-N1 to construct $N36_{mCAT1}$-GFP and $N18_{mCAT1}$-GFP. Sequences encoding the N-terminal 239, 182, 124, and 61 amino acids of mCAT1 were amplified by PCR. The upstream primers for these four constructs were the same: 5′-CCG GAA GCG GCC ATG GGC TGC AAA AAC CTG CTC GGT-3′. The downstream primers were: 5′ CCG GTC CGG ACC CTC CCT CAC CGT ATT TCA CGT T-3′, 5′-CCG GTC CGG AGT

TGT TGT TAC AGG AGA AAT-3′, 5′-CCG GTC CGG AAC CGA TCA TGT AGG AGA GAA T-3′, and 5′-CCG GTC CGG AAT TTT CAC GGG CCA CGG CAC C-3′, respectively. The PCR products were treated with Klenow large fragment in the presence of 0.2 mM dATP and dTTP for 15 min at 25°C to create *Age*I overhangs, and then ligated into pEGFP-N1 (Clontech) that was cut with *Age*I and dephosphorylated, yielding $N239_{mCAT1}$-GFP, $N182_{mCAT1}$-GFP, $N124_{mCAT1}$-GFP, and $N61_{mCAT1}$-GFP. $N18(RR\text{-}EE)_{mCAT1}$-GFP, containing glutamic acid instead of arginine at positions 15 and 16, was made by ligating the following oligonucleotide adapters into the *Age*I site of pEGFP-N1 (bold indicates codons encoding glutamic acid; an *Xho*I site used to identify correct clones is underlined): 5′-CCG GCC GCC ATG GGC TGC AAA AAC CTG CTC GGT CTG GGC CAG CAG ATG CTC **GA**G **GAG** AAG GTG-3′ and 5′-CCG GCA CCT TCT C*CT CGA G*CA TCT GCT GGC CCA GAC CGA GCA GGT TTT TGC AGC CCA TGG CGG-3′. A plasmid encoding the first 18 amino acids of human CAT1 fused to the N-terminus of GFP ($N18_{hCAT1}$-GFP) was made by PCR site-directed mutagenesis of the plasmid encoding mouse $N18_{mCAT1}$-GFP using a kit (Stratagene, Cedar Creek, TX). The mutagenesis primers were 5′-GGG CTG CAA AGT CCT GCT CAA TAT TGG CCA GCA GAT GC-3′ and 5′-GCA TCT GCT GGC CAA TAT TGA GCA GGA CTT TGC AGC CC-3′. The *Msc*I site (underlined) was created for clone identification. To construct MTP-DsRed2, the mitochondrial-targeting sequence of pEYFP-mito (Clontech) was excised with *Age*I/*Not*I and inserted into pDsRed2-N1 (Clontech) cut by the same enzymes. $N18_{mCAT1}$-GFP-mCAT1 was created by replacing GFP in GFP-mCAT1 by $N18_{mCAT1}$-GFP cut with *Bsr*GI/*Nhe*I. To construct MTP-mCAT1(Δ4–33)-GFP, mCAT1(Δ4–33)-GFP was excised from the Sindbis expression vector using *Xba*I and *Not*I and ligated into pEYFP-Mito (Clontech) cut with *Bam*HI and *Not*I, using adaptors 5′-GATCGGCGCGCC-3′ and 5′-CTAGGGCGCGCC-3′ to convert the *Bam*HI overhang to an *Xba*I overhang.

In vitro transcription and electroporation

All constructs in Sindbis expression vectors were linearized with *Not*I and transcribed in vitro following the manufacturer's protocol (Invitrogen, Carlsbad, CA). For electroporation, 2×10^6 BHK or XC cells were resuspended in 400 μl of serum-free PBS buffer containing in vitro-transcribed RNA and placed in a 0.4-cm cuvette (Bio-Rad Laboratories, Hercules, CA). Cells were subjected to two 600-V pulses at 25 μF, yielding a typical time constant of about 0.7 ms in a Bio-Rad Gene Pulser (Bio-Rad Laboratories). Electroporated cells were then placed on ice several min to recover, diluted in DMEM medium supplemented with 10% serum, 1% glutamine, and 1% antibiotics, and seeded in 8-well coverglass chambers (Nunc, Germany).

LipofectAMINE transfection

LipofectAMINE 2000 was purchased from Invitogen. Plasmid DNA and liposomes were mixed according to the manufacturer's protocol. Briefly, 0.5 μg DNA and 1 μl liposome were mixed in 100 μl OPTI-Mem medium (Gibco, Frederick, MD) for 30 min at room temperature. Monolayer HEK 293, BHK, or XC cells about 80% confluent were washed with serum-free DMEM medium and OPTI-Mem, incubated with the DNA-liposome mixture in a CO_2 incubator for 3–6 h at 37°C, and then refed with fresh culture medium for another 12–24 h.

Mitochondria staining with MitoTracker Red and antibody to cytochrome oxidase

Monolayer cells were incubated for 15 min with 100 nM Mitotracker Red (Molecular Probes, Eugene, OR) in phosphate-buffered saline containing calcium and magnesium (PBS^+) and washed with PBS^+. For cytochrome oxidase labeling, monolayer cells were washed with PBS, fixed for 20 min with methanol precooled to −20°C, and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. After permeabilization, the cells were washed again and then incubated in blocking buffer (3% bovine serum albumin in PBS, pH 7.4) for 30 min at room temperature. The primary antibody, anti-cytochrome oxidase subunit I (Molecular Probes), diluted 1:50 in blocking buffer, was then added for 1 h at room temperature. After washing, Cy5-conjugated anti-mouse IgG (1:50, Jackson ImmunoResearch, West Grove, PA) was added for 1 h at room temperature, followed by washing with PBS.

Syncytium formation assay

BHK cells transfected with mCAT1 or mCAT1 mutants were cocultured with indicator BHKenv cells (Kazachkov et al., 2000), which express a fusogenic form of the envelope of ecotropic murine leukemia virus, and incubated at 37°C in a 5% CO_2 incubator. Cells were fixed and stained 12–24 h later with methanol containing 0.17% (wt/vol) carbol fuchsin and 0.5% methylene blue. Plain BHK cells were cocultured with BHKenv cells as a negative control.

Confocal fluorescence microscopy

Cells were plated in 8-well coverglass chambers (Nunc, Germany) and examined with a Leica TCS-NT/SP confocal microscope (Leica, Wetzlar, Germany) using a 63x objective and Omnichrome argon-krypton lasers emitting at 488, 568, and 647 nm. In colocalization studies, images were recorded with simultaneous excitation and detection of both dyes. Cross-talk was eliminated by adjusting the laser intensity, detection wavelength windows, and photodetector

sensitivities so that no signal was detected in the red channel when the sample was excited with the green laser alone, and vice versa.

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